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## **Ultra-deep sequencing confirms immunohistochemistry as a highly sensitive and specific method for detecting BRAF (V600E) mutations in colorectal carcinoma**

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# Ultra-deep sequencing confirms immunohistochemistry as a highly sensitive and specific method for detecting *BRAF*<sup>V600E</sup> mutations in colorectal carcinoma

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**Abstract** The activating *BRAF*<sup>V600</sup> mutation is a well-established negative prognostic biomarker in metastatic colorectal carcinoma (CRC). A recently developed monoclonal mouse antibody (clone VE1) has been shown to detect reliably *BRAF*<sup>V600E</sup> mutated protein by immunohistochemistry (IHC). In this study, we aimed to compare the detection of *BRAF*<sup>V600E</sup> mutations by IHC, Sanger sequencing (SaS), and ultra-deep sequencing (UDS) in CRC. VE1-IHC was established in a cohort of 68 *KRAS* wild-type CRCs. The VE1-IHC was only positive in the three patients with a known *BRAF*<sup>V600E</sup> mutation as assessed by SaS and UDS. The test cohort consisted of 265 non-selected, consecutive CRC samples. Thirty-nine out of 265 cases (14.7 %) were positive by VE1-IHC. SaS of 20 randomly selected IHC negative tumors showed *BRAF* wild-type (20/20). Twenty-four IHC-positive cases were confirmed by SaS (24/39; 61.5 %) and 15 IHC-positive cases (15/39; 38.5 %) showed a *BRAF* wild-type by SaS. UDS detected a *BRAF*<sup>V600E</sup> mutation in 13 of these 15 discordant cases. In one tumor, the mutation frequency was below our threshold for UDS positivity, while in another case, UDS could not be performed due to low DNA amount. Statistical analysis showed sensitivities of 100 % and 63 % and specificities of 95 and 100 % for VE1-IHC and SaS, respectively, compared to

combined results of SaS and UDS. Our data suggests that there is high concordance between UDS and IHC using the anti-*BRAF*<sup>V600E</sup> (VE1) antibody. Thus, VE1 immunohistochemistry is a highly sensitive and specific method in detecting *BRAF*<sup>V600E</sup> mutations in colorectal carcinoma.

**Keywords** *BRAF* mutation · Immunohistochemistry · Sanger sequencing · Ultra-deep sequencing · Colorectal carcinoma

## Introduction

Colorectal carcinoma (CRC) is the third most malignant neoplasm in males and second most in females worldwide [1]. In a metastasized stage, the 5-year survival rate decreases to less than 10 % [2]. A combined systemic therapy including cetuximab, a monoclonal anti-EGFR antibody, increases the overall and progression-free survival in tumors bearing a wild-type of *KRAS* ('Kirsten rat sarcoma viral oncogene homolog') and *BRAF* ('v-raf murine sarcoma viral oncogene homolog B1') genes [3, 4]. In contrast, mutated *BRAF* is a marker of worse prognosis in CRC [5–7]. The most common genetic change in the *BRAF* gene of CRC is a c.1799 T > A point mutation in Exon 15, causing an amino acid exchange from valine to glutamic acid (p.V600E), which leads to a 10-fold increased kinase activity [8–10].

Usually, analysis of the *BRAF*<sup>V600E</sup> status is performed using different polymerase chain reaction (PCR)-based detection methods like Sanger sequencing, pyrosequencing, and qPCR. Besides the average costs of approximately \$300 per analysis [11, 12], these techniques are time consuming and require a high-quality laboratory infrastructure with well-trained staff. Currently, Sanger sequencing is probably the most often used technique because of its reliability and high specificity.

Matthias Rössle and Michèle Sigg contributed equally to this study.

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However, its sensitivity usually does not allow detecting mutations below a frequency of 15–20 % tumor cells [13, 14].

Among other more sensitive techniques, next-generation sequencing technology overcomes this disadvantage and reveals low-frequency mutations in heterogeneous tumor populations. Recently, we demonstrated the reliability of this technique as a standard procedure in diagnostics of CRC using genomic DNA isolated from formalin-fixed paraffin-embedded tissues (FFPE) [15].

In contrast, immunohistochemistry is a tissue-based, cost-effective technique, which is easy to perform and routinely available in most pathology laboratories [16]. Capper et al. [17] developed a monoclonal mouse antibody (clone VE1), which recognizes the *BRAF*<sup>V600E</sup> protein. Several studies have shown that this antibody reliably detects *BRAF*<sup>V600E</sup> mutations in malignant neoplasms by immunohistochemistry in routinely processed FFPE tissues [18–26].

In our study, we aimed to evaluate the reliability of this new antibody in detecting *BRAF*<sup>V600E</sup> mutations compared to conventional Sanger sequencing in a cohort of consecutive CRCs. Furthermore, we aimed to assess the sensitivity of the antibody below the detection limit of Sanger sequencing by including ultra-deep sequencing (UDS).

## Materials and methods

### Patients

For this study, tumor tissue, and tissue microarray (TMA) slides from two cohorts were analyzed. We have previously described a cohort of 68 *KRAS* wild-type CRCs [15]. Among these 68 *KRAS* wild-type CRCs, we identified three tumors with *BRAF*<sup>V600E</sup> mutations as assessed by Sanger sequencing (SaS) and UDS. All cases were diagnosed at the Institute of Surgical Pathology, University Hospital Zurich (Switzerland) between 2006 and 2011. The median age was 61 ranging from 35 to 83. Nineteen patients were females and 49 males.

The test cohort consisted of non-selected, consecutive CRC samples of 265 patients, diagnosed at the Institute of Surgical Pathology, University Hospital Zurich between 2001 and 2011. The age ranged from 21 to 95 years (median 72 years). Both sexes were equally distributed (133 females, 132 males). The study was approved by the Cantonal Ethics Committee of Zurich (KEK-ZH-NR: 2010-0093/0).

### Tissue microarray construction

Original hematoxylin and eosin stained sections of all CRC samples were retrieved from the archives of the Institute of Surgical Pathology, University Hospital Zurich and reviewed by two histopathologists (antibody establishment cohort: J.R., H.M.; test cohort: M.S., A.W.). The TMAs were constructed

with core replicas as described elsewhere [27]. In brief, two tissue cores (diameter 0.6 mm) of a representative tumor area of each patient were taken from a “donor” block and arranged in a new “recipient” block using a custom-built instrument. Each recipient block also included 14 colon cancer cell lines with known *BRAF* mutational status (six cell lines with a *BRAF*<sup>V600E</sup> mutation, eight cell lines with *BRAF* wild-type), which served as controls. Recipient blocks of the test cohort additionally include normal colorectal tissue of CRC patients as additional control tissue.

### Immunohistochemistry

Immunohistochemistry (IHC) was conducted on 2-μm thick sections of both TMAs with Ventana Benchmark XT automated staining system (Ventana Medical Systems, Tucson, AZ, USA) and according to the manufacturer's instructions. Briefly, after antigen retrieval with cell conditioner 1 (Ventana Medical Systems) for 64 min and pre-primary peroxidase inhibition, the TMA slides were incubated with *BRAF*<sup>V600E</sup> antibodies (clone VE1, dilution 1:200; Spring Bioscience, Pleasanton, USA) for 32 min at 37 °C. After that, incubation with OptiView DAB IHC Detection Kit (Ventana Medical Systems) followed. Slides were counterstained with hematoxylin and Bluing reagent for 4 min each. The reaction quality was controlled using cell lines on the TMAs with known *BRAF*<sup>V600E</sup> mutational status. The immunostained slides were independently evaluated by two pathologists (M.S. and M.Rö.), both blinded to the *BRAF* mutation status. TMA spots lacking of tumor tissue were excluded from the analysis. According to the unequivocal cytoplasmic staining of a majority of the tumor cells, TMA spots were ranked into three staining categories: score 0 for negative, score 1 for weakly/moderately positive, and score 2 for strongly positive. Of each case, the strongest immunoreactivity of the two spots was counted.

### DNA extraction

Three tissue cylinders (diameter 0.6 mm) were punched from formalin-fixed, paraffin-embedded (FFPE) tissue block of each CRC patient. After extraction of genomic DNA using DNeasy Blood & Tissue Kit 250 (Qiagen, Hilden, Germany), DNA-quantification was done with NanoDrop.

### Sanger sequencing

Sanger sequencing evaluating the mutational status of *BRAF* exon 15 was done for all IHC-positive and for 20 IHC-negative cases of the test cohort. DNA was PCR amplified (AmpliTaQ Gold, Roche, Switzerland) using the primers described in Table 1. The cycling conditions were 5 min at 95 °C, 40 cycles each with 1 min at 95 °C, 1 min at 53 °C, 1 min at 72 °C and a final elongation step of 10 min at 72 °C.

**Table 1** Primers used for Sanger sequencing and ultra-deep sequencing of *BRAF* exon 15

Sequencing	Primer
Sanger sequencing	
<i>BRAF</i> Ex.15 F	5'-TCATAATGCTTGCTCTGATAGGA-3'
<i>BRAF</i> Ex.15 R	5'-GGCCAAAAATTTAATCAGTGGA-3'
Ultra-deep sequencing <sup>a</sup>	
<i>BRAF</i> 15 F_MID1	5'-CGTATCGCCTCCCTCGCGCCATCAGACGAGTGCGTTCATAATGCTTGCTCTGATAGGA-3'
<i>BRAF</i> 15 R_MID1	5'-CTATGCGCCTTGCCAGCCCGCTCAGACGAGTGCGTGCCAAAAATTTAATCAGTGGA-3'
<i>BRAF</i> 15 F_MID2	5'-CGTATCGCCTCCCTCGCGCCATCAGACGCTCGACATCATAATGCTTGCTCTGATAGGA-3'
<i>BRAF</i> 15 R_MID2	5'-CTATGCGCCTTGCCAGCCCGCTCAGACGCTCGACAGGCCAAAAATTTAATCAGTGGA-3'
<i>BRAF</i> 15 F_MID3	5'-CGTATCGCCTCCCTCGCGCCATCAGACGCACTCTCATAATGCTTGCTCTGATAGGA-3'
<i>BRAF</i> 15 R_MID3	5'-CTATGCGCCTTGCCAGCCCGCTCAGACGCACTCTGGCCAAAAATTTAATCAGTGGA-3'
<i>BRAF</i> 15 F_MID4	5'-CGTATCGCCTCCCTCGCGCCATCAGACACTGTAGTCATAATGCTTGCTCTGATAGGA-3'
<i>BRAF</i> 15 R_MID4	5'-CTATGCGCCTTGCCAGCCCGCTCAGACACTGTAGGGCCAAAAATTTAATCAGTGGA-3'

Ex exon, F forward, R reverse

<sup>a</sup> Italic letters in primers: ultra-deep sequencing adapters with multiplex identifiers (MIDs)

PCR products were purified using the Qiagen MiniElute PCR Purification Kit (Qiagen) and sequenced with a Genetic Analyzer 3130xl (Applied Biosystems) using the same primers mentioned above. A change of the electropherogram was classified as mutation, if the peak of the aberrant base (i) was clearly above background, (ii) was present in forward and reverse direction, and (iii) its height exceeded more than 10 % of the reference base peak height. Examples for Sanger sequencing declared as wild-type are shown in Supplementary Figure S1. As a comparison of a “true” mutation, co40 is depicted as positive control in Supplementary Figure S1.

#### Ultra-deep sequencing (UDS)

Additional UDS was performed to assess the *BRAF* status in all cases of the test cohort, which showed discordant results after IHC and Sanger sequencing. The UDS procedure is described elsewhere [15]. Shortly, an independent PCR with fusion primers including multiplex identifiers (MIDs) (Table 1) was performed from the same DNA sample used for Sanger sequencing. Amplicon processing was done as described by the Amplicon Library Preparation and emPCR (Lib-A) Method GS Junior Titanium Series manual from Roche. 500,000 enriched beads were loaded on a 454 Junior Sequencer (Roche). Demultiplexing and variant calling was done with the Amplicon Variant Analyzer v2.7 (AVA) software from Roche. Selection criteria (at least 50 reads containing the mutation and at least 0.38 % mutation frequency) for a true positive variant are described in Rechsteiner et al. [15].

#### Statistics

The calculation of the IHC VE1 sensitivity, specificity, and positive (PPV) and negative predictive value (NPV) including

their 95 % confidence intervals (CI) by comparison with the combined results of both sequencing methods as “gold standard” was performed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, USA). Measurement of interobserver agreement was performed using SPSS 20.0 (IBM Corporation, Armonk, USA).

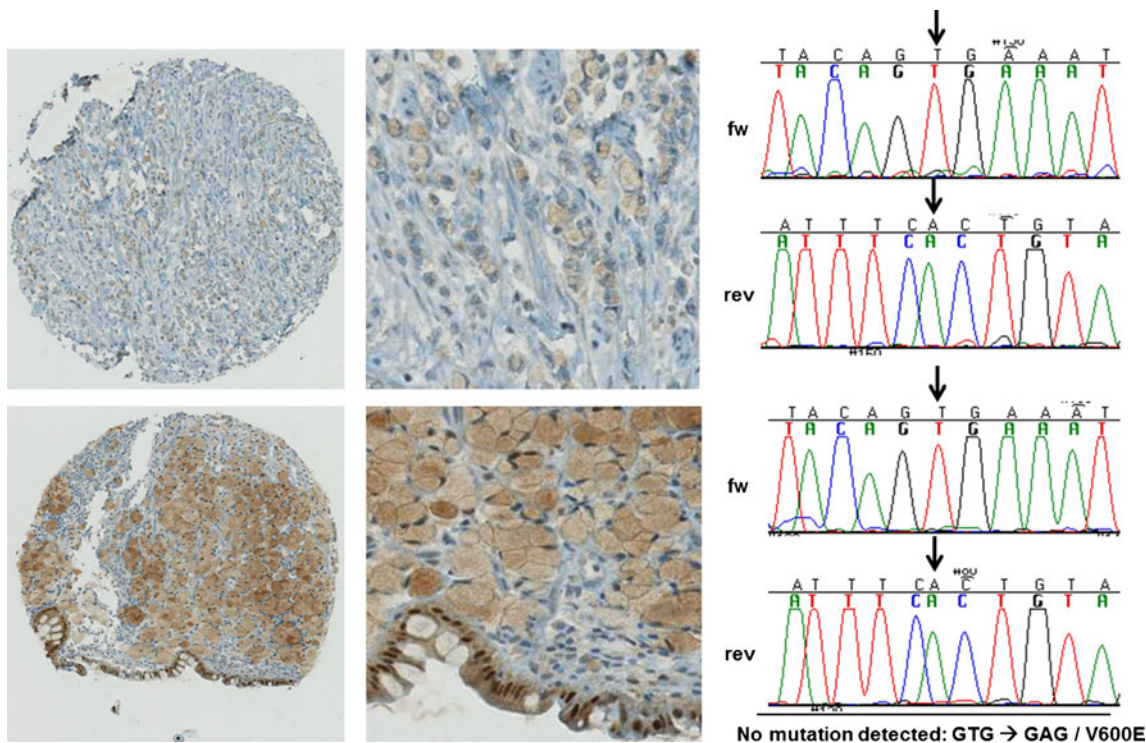
## Results

### *BRAF*<sup>V600E</sup> (VE1) antibody establishment

Immunohistochemistry of cell line controls showed moderate positivity in one and strong positivity in five of the six known *BRAF*<sup>V600E</sup> mutated cell lines, while all eight cell lines with known *BRAF* wild-type were immunohistochemically completely negative. A representative staining of the cell lines is shown in Supplementary Figure S2.

The *BRAF*<sup>V600E</sup> (VE1) antibody was further established in 68 *KRAS* wild-type CRCs with three (4.4 %) *BRAF*<sup>V600E</sup> mutations previously identified by SaS and UDS. All three *BRAF*<sup>V600E</sup> mutated CRC were immunohistochemically positive and showed strong staining reactivity with the VE1 antibody. Some CRCs showed an uncharacteristic weak to strong staining in the intracellular mucus of signet ring-like cells. In the upper row of Fig. 1, one CRC with such a staining pattern is depicted with its Sanger sequence on the right panel without visible mutation. Another CRC with strong staining is presented in the lower row of Fig. 1 with its corresponding sequence. Due to the fact that no *BRAF* mutation could be detected in both CRCs by sequencing, all similar stainings in the intracellular mucus of signet ring-like cells were classified as artifacts and scored negative. All other cases were unequivocally negative, resulting in a sensitivity





**Fig. 1** Unspecific staining of tissue by VE1-IHC. Moderate (score 1, upper row) and strong (score 2, lower row) staining of intracellular mucus in signet ring-like cells (original magnifications: left panel×10, middle

panel×40) and nuclei of normal surface epithelium. The right panel shows the corresponding wild-type Sanger sequences. Arrows indicate the location of the T/A (forward strand) mutation leading to *BRAF*<sup>V600E</sup>

and specificity of 100 % each. There was a perfect interobserver agreement with a kappa value of 1.000.

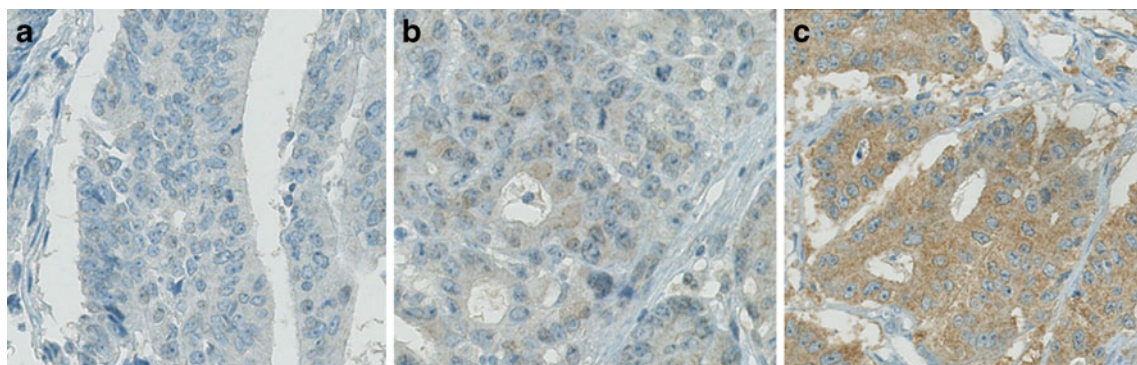
#### VE1 expression in colorectal carcinoma of the test cohort

Both observers agreed on the interpretation of the VE1 immunohistochemical staining in all but five cases (three cases: score 0 vs. score 1; two cases: score 1 vs. score 2) resulting in a kappa value of 0.921 ( $p < 0.01$ ). After discussion, a consensus was obtained. One case was not evaluable because of loss of

tumor material during staining process. Of the remaining 264 cases, 225 (85.2 %) were immunohistochemically negative, 19 (7.2 %) showed an IHC-score 1, and 20 (7.6 %) an IHC-score 2 (Fig. 2).

#### *BRAF* mutational analysis by Sanger sequencing in colorectal carcinoma of the test cohort

These 39 positive cases as well as 20 randomly selected IHC-negative tumors were submitted to *BRAF*<sup>V600E</sup> mutation



**Fig. 2** VE1 immunohistochemistry of different colorectal carcinoma of the test cohort with score 0 (a×40 original magnification), score 1 (b×40), and score 2 (c×40) for VE1-immunohistochemistry

analysis. SaS of the 20 randomly chosen IHC-negative cases confirmed the immunohistochemical results and showed no mutation in exon 15 of the *BRAF* gene. Of the 19 IHC-score 1 cases, SaS showed a *BRAF*<sup>V600E</sup> mutation in 13 cases and a *BRAF* wild-type situation in six tumors. Among the 20 IHC-score 2 cases, SaS resulted in 11 *BRAF*<sup>V600E</sup> mutated and in nine wild-type cases (Fig. 3).

#### *BRAF* mutational analysis by Ultra-deep sequencing in colorectal carcinoma of the test cohort

UDS was performed in 14 IHC-positive cases, which were *BRAF* wild-type by SaS. The DNA amount was too low for analysis in one case. Additionally, one case with IHC-score 1 in one TMA core (site 1) and IHC-score 2 on the second TMA core (site 2) was also included in the UDS analysis. Interestingly, DNA extraction from site 1 and subsequent SaS detected a *BRAF* mutation in the core with IHC-score 1 and none in the DNA extracted from site 2. Therefore, UDS was performed with the DNA derived from site 2 to investigate whether a low frequency mutation was missed by SaS. Moreover, a positive control was included in the UDS analysis, in which SaS detected a *BRAF*<sup>V600E</sup> mutation.

In Fig. 4a, the total number of reads from the UDS analysis, including mutated and wild-type *BRAF* sequences, is shown per patient (mean 7,077 reads). In Fig. 4b, the fraction of reads with *BRAF*<sup>V600E</sup> mutations is depicted (mean 449 reads). In 14 cases, a *BRAF*<sup>V600E</sup> mutation was detected by UDS with mutation frequencies ranging from 1.85–16.36 % (Fig. 4c). Importantly, a mutation with a frequency of 1.85 % was detected in the DNA extracted from site 2 of the abovementioned

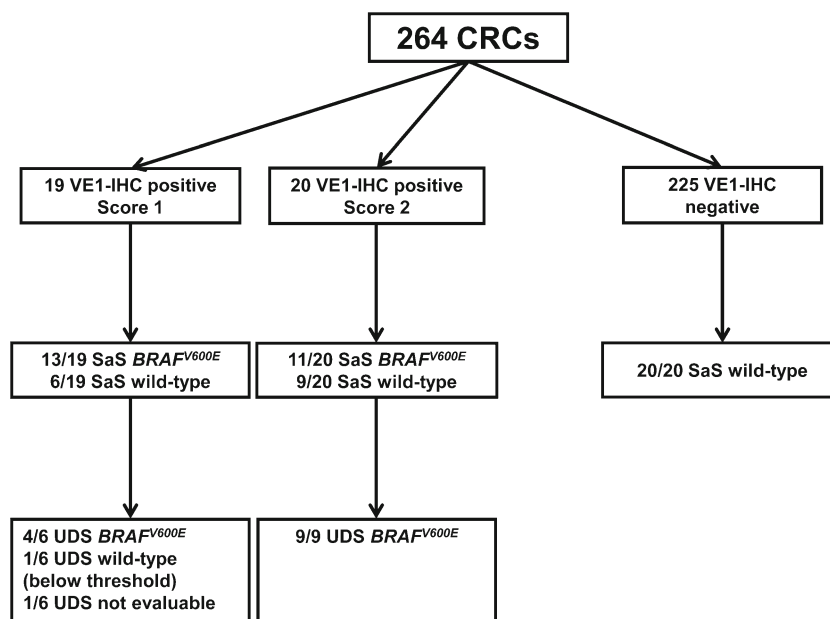
case underscoring the reliability of the VE1 staining. In the remaining case, a mutation frequency of 0.79 % with only 29 reads containing the mutation was identified which, however, was below our UDS threshold to be included as a true variant (Fig. 4c). The positive control (co40) was successfully detected at high frequency (29 %).

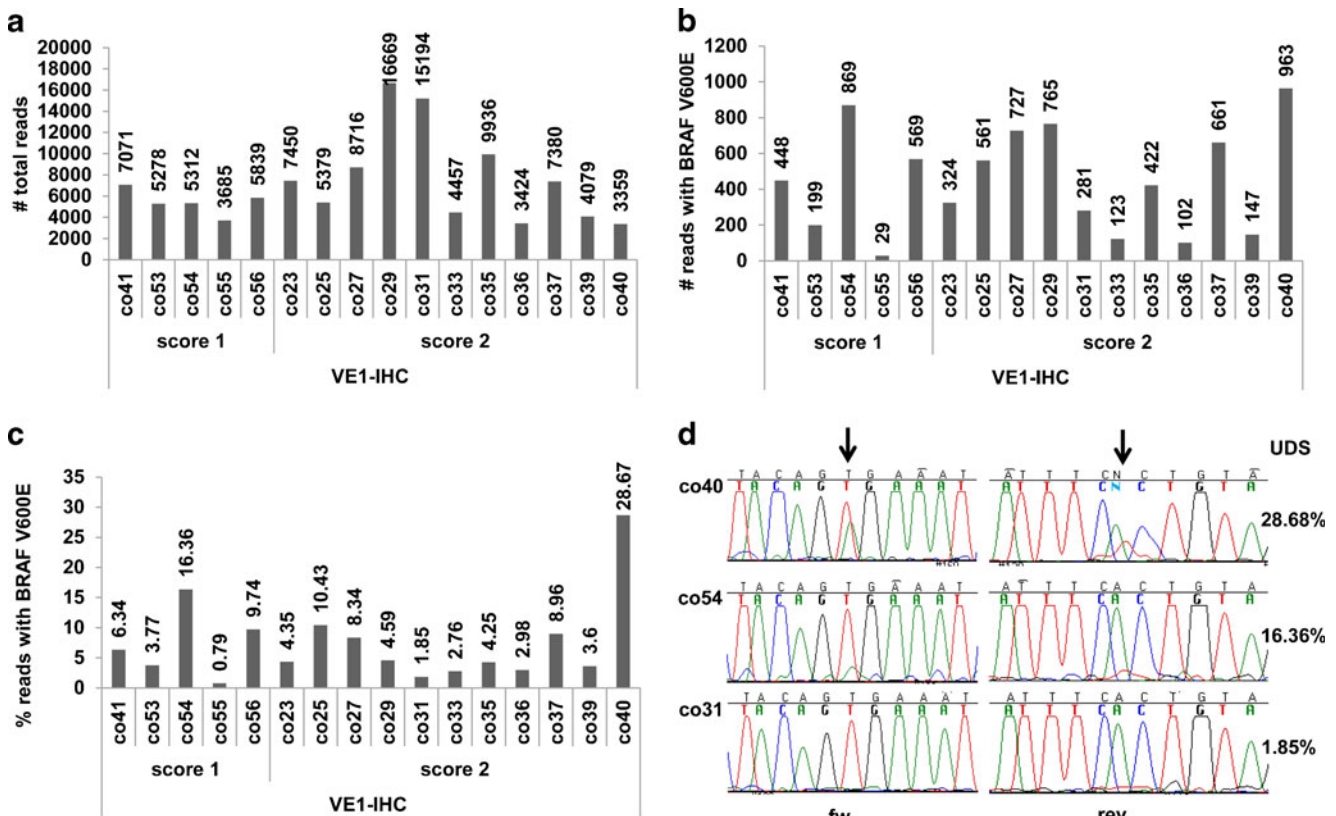
#### Comparison of results of IHC, SaS, and UDS in colorectal carcinoma of the test cohort

As a comparison of VE1-IHC, SaS, and UDS, these 15 cases and the positive control are shown in the Supplementary Figure S1. The mutated base peak height from SaS was comparable to the percentage of the mutation found by UDS. However, all cases except for the positive control were classified as wild-type when using SaS according to our mutation detection threshold level explained in the “Materials and methods” section. In contrast, SaS and UDS results did not correlate in all cases with the VE1-IHC staining. This is mainly due to the heterogeneity of the tissue (tumor/stromal/normal cells), which is shown for one case (co25; Fig. 5). In this case, the TMA punches gave an IHC-score 2 which matches the TMA extraction cores of the whole tissue cut in Fig. 5. In contrast, the punches taken for DNA extraction included one punch at a location with low tumor content which might have resulted in the wild-type classification by SaS and in the low percentage of mutated reads by UDS (10.43 %).

Together, SaS and UDS detected *BRAF*<sup>V600E</sup> mutations in 37 (14 %) and *BRAF* wild-type in 22 tumors (Fig. 3). The case with VE1-IHC positivity and a *BRAF* mutation below the

**Fig. 3** Flow chart of immunohistochemistry (IHC), Sanger sequencing (SaS), and ultra-deep sequencing (UDS) on test cohort





**Fig. 4** Ultra-deep sequencing (UDS) of selected colorectal carcinoma cases negative for *BRAF*<sup>V600E</sup> by Sanger sequencing but positive by VE1-IHC. *co40*: positive control with a *BRAF*<sup>V600E</sup> mutation detected by Sanger sequencing. **a** Total read count of wild-type and *BRAF*<sup>V600E</sup> mutated

sequences. **b** Reads with *BRAF*<sup>V600E</sup> mutations. **c** Percentage of *BRAF*<sup>V600E</sup> mutated reads compared to total reads. **d** Sanger sequences of *co40*, *co54*, and *co31* with *BRAF*<sup>V600E</sup> mutations of 28.68, 16.36, and 1.85 % mutation frequencies, respectively, assessed by UDS

detection limit of UDS (29 reads) was counted as false positive and included in the wild-type cases. The case with negative SaS and missing subsequent UDS was not counted for further statistical analysis.

Statistical analysis showed a sensitivity of 100 % (95 % CI 90.75–100.00 %) and a specificity of 95.24 % (95 % CI 76.18–99.88 %) for VE1-IHC compared with combined results of both SaS and UDS as “gold standard” (see Table 2). The PPV was 97.44 % (95 % CI 86.52–99.94 %) and the NPV was 100.0 % (95 % CI 83.16–100.0 %) for VE1-IHC.

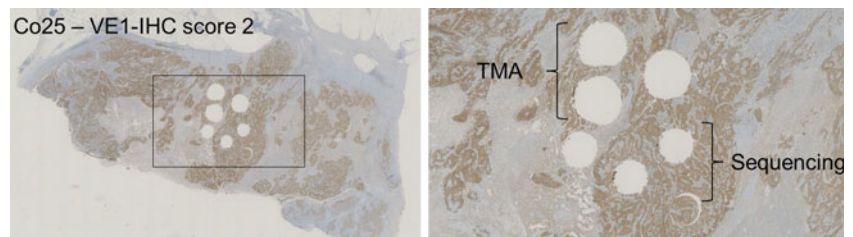
For SaS compared with combined results of both sequencing methods (see Table 3) a sensitivity of 63.16 % (95 % CI 45.99–78.19 %), a specificity of 100.0 % (95 % CI 83.16–100.00 %),

a PPV of 100.0 % (95 % CI 85.75–100.00 %), and a NPV of 62.86 % (95 % CI 40.7–75.35 %) was calculated.

## Discussion

Our results show a very high reliability of the VE1 immunohistochemistry detecting *BRAF*<sup>V600E</sup> mutations in colorectal carcinoma with a sensitivity of 100 % and a specificity of 95.24 %. Similar results were obtained in previous studies using the VE1 antibody on different tumors like malignant melanoma [24, 28], Langerhans cell histiocytosis [26], hairy cell leukemia [18], papillary thyroid carcinoma [20, 23],

**Fig. 5** Tissue heterogeneity of VE1-IHC stained colon carcinoma tissue. *Left panel*: whole tissue section. *Right panel*: big cores taken for TMA construction and smaller cores punched for DNA extraction and sequencing





**Table 2** Comparing results of VE1-immunohistochemistry with combined results of Sanger and ultra-deep sequencing for *BRAF*<sup>V600E</sup> mutations

		Combined sequencing results		Total
		Negative	Positive	
VE1-immunohistochemistry	Negative	20	0	20
	Positive	1	37	38
Total		21	37	58

epithelial ovarian tumors [19, 25], and pulmonary adenocarcinoma [22].

The almost perfect interobserver agreement shows that the interpretation of immunohistochemical staining results is quite clear and simple in most cases. Using the well-defined criteria (“diffuse and homogeneous granular cytoplasmic staining of tumor cells”) for positivity, only few pitfalls, which have been partly described previously [20, 21, 29, 30], occur. In colorectal tissues, sometimes, a nuclear staining of normal epithelial cells as well as an unspecific positivity of the intracellular mucus of goblet cells and signet ring-like tumor cells can be observed. Additionally, tumor-associated macrophages that sometimes show a cytoplasmic granular positivity may be difficult to differentiate from true-positive tumor cells.

According to the COSMIC database and literature, *BRAF* mutations are found in about 4–16 % of CRC [3, 13, 31], which fits very well with the frequency in our test cohort bearing in mind that *BRAF*<sup>V600E</sup> changes counts for more than 80 % of all *BRAF* mutations in CRC [9]. As we performed Sanger sequencing only in 20 out of 225 VE1-IHC-negative cases, we cannot exclude that some of the remaining IHC-negative cases might have low frequency or other *BRAF* mutations, as well, than *BRAF*<sup>V600E</sup>. However, the aim of this study was rather to assess the detection limit of the VE1 antibody than the detection limit of our UDS approach. The lower rate of *BRAF*<sup>V600E</sup> mutations in our antibody establishment cohort (4.4 %) can be explained by a selection bias, while in contrast our test cohort consisted of non-selected, consecutive colorectal carcinoma specimen.

Our study shows some limitations of Sanger sequencing (SaS) in detecting *BRAF* mutations. It is well known that for

successful SaS, a minimal tumor cell amount of 10–20 % is necessary. In summary, four (all VE1-IHC-score 2) out of 15 Sanger-negative cases had a tumor cell amount on TMA cores below 30 %. Three ones (all VE1-IHC-score 1), including that one on which UDS could not be performed and that one with UDS negativity due to low number of positive reads, showed a heterogeneous VE1-IHC staining pattern. In a recent study on mismatch repair protein-deficient colorectal carcinomas, Capper et al. also reported one case with a high amount of non-tumorous cells, which showed positive VE1-IHC and negative Sanger sequencing and pyrosequencing [32]. However, in eight cases with homogeneous VE1-IHC positivity (three with score 1, five with score 2) and a tumor cell percentage >60 %, SaS failed repeatedly to detect the *BRAF* mutation. Thus, despite the fact that we used tumor punches instead of whole sections for DNA extraction, which usually results in a higher tumor cell load, SaS failed in 38 % of our cases to detect the *BRAF*<sup>V600E</sup> mutation. One critical aspect of punching the tumor area for DNA extraction is to allocate and to hit the right area for punching. Such a case is shown in Fig. 5, where one punch at a location with low tumor content was included. This punch might have resulted in the wild-type classification by SaS and in the low percentage of mutated reads by UDS. Another case reflecting the same problem was investigated by comparing sequencing data obtained from DNA extracted from two different sites of a tumor which both were positive for VE1-IHC. SaS detected a *BRAF*<sup>V600E</sup> mutation only in one of these two sites. Subsequent UDS of the Sanger-negative material revealed the same mutation at low frequency. It may be speculated if VE1-IHC, which can also highlight single tumor cells, was more sensitive detecting the *BRAF* mutation than SaS or if VE1-IHC was false positive in these tumor cells. The fact that UDS detected the mutation at low frequency rather suggests a higher sensitivity of VE1-IHC. Whether, in this case, the low mutation frequency was due to the contamination of normal cells or due to intratumoral heterogeneity remains elusive. Assuming the latter variant, these results might reflect true intratumoral heterogeneity of *BRAF* mutation, which has been reported as rare events (1–3 %) in primary CRC [33, 34].

Another reason why, in our study, 38 % of the *BRAF* mutations were missed by SaS might be the stringent selection criteria we applied for classifying a mutation as true by SaS. Retrospectively, one might adjust the detection threshold as in most cases of the 15 previously classified wild-type cases a mutated base peak was visible in SaS.

Our results agree with previous studies comparing SaS with other sensitive detection methods like pyrosequencing or deep sequencing. Guerra et al. reported a similar result comparing *BRAF* mutational status of macrodissected papillary thyroid carcinoma by SaS and pyrosequencing [35]. Thus, VE1-IHC seems to be sensitive enough to overcome the problem of tissue and tumor heterogeneity. Taken together,

**Table 3** Comparing results of Sanger sequencing alone with combined results of Sanger and ultra-deep sequencing for *BRAF*<sup>V600E</sup> mutations

		Combined sequencing results		Total
		Negative	Positive	
Sanger sequencing	Negative	20	14	34
	Positive	0	24	24
Total		20	38	58



we demonstrate the high sensitivity and specificity of detecting *BRAF*<sup>V600E</sup> mutations in colorectal carcinoma by a new mutation-specific antibody. Our IHC results agree with two other recently published studies investigating the VE1 antibody on CRC specimens [29, 36]. Both groups reported a high concordance between VE1-IHC and pyrosequencing [29] or multiplex allelic-specific PCR-based assay [36] as reference methods. In contrast, a study by Adackapara et al. [30] showed a much lower sensitivity (35 %) for VE1-IHC on CRC specimen compared to pyrosequencing results. They used a manual technique with overnight incubation for VE1-IHC. Therefore, methodological differences could explain the difference to ours and other studies.

Our study represents the first comparison between UDS and VE1-IHC in CRC and we demonstrate a very high concordance between UDS and VE1-IHC. In the light of the relatively high costs and demanding infrastructure of ultra-deep sequencing as well as the lower sensitivity of SaS, the VE1-IHC, which can be performed routinely in probably most pathological institutes, seems to be a reliable, simple, and cheap primary tool to analyze *BRAF*<sup>V600E</sup> status for diagnostic, predictive, and prognostic purposes. Only VE1-negative or equivocal cases may be further investigated by more sensitive methods like ultra-deep sequencing.

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**Conflict of interests** The authors declare that they have no conflicts of interest.

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